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Improved Detection of Respiratory Viruses in Pediatric Outpatients with Acute Respiratory Illness by Real-Time PCR Using Nasopharyngeal Flocked Swabs▽

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Detection of respiratory viruses by real-time multiplexed PCR (M-PCR) and of respiratory syncytial virus (RSV) by M-PCR and immunofluorescence (IF) was evaluated using specimens collected by nasopharyngeal flocked swabbing (NFS) and nasal washes (NW). In children with mild respiratory illness, NFS collection was superior to NW collection for detection of viruses by M-PCR (sensitivity, 89.6% versus 79.2%; $P = 0.0043$). NFS collection was noninferior to NW collection in the detection of RSV by IF.

Respiratory viruses are major causes of infant and childhood acute respiratory infection (ARI), with respiratory syncytial virus (RSV) contributing significantly to the disease burden (3, 5, 11). Nasopharyngeal aspiration (NPA) and nasal washes (NW) have been the preferred sampling methods for the diagnosis of respiratory viruses (7, 12, 13). However, nasopharyngeal flocked swabbing (NFS) is increasingly recognized as an alternative (1). Possible advantages of NFS over NW and NPA include simplicity of use, improved standardization in different age groups and between operators, and better acceptability in a wider range of settings. However, there is little published data on the sensitivity of NFS when using real-time multiplex PCR (M-PCR) assays for virus detection (10). We report on a study designed to assess the diagnostic performance of NFS relative to NW for detection of RSV by both immunofluorescence (IF) antibody tests and M-PCR and for other respiratory viruses by M-PCR only.

In an outpatient health facility serving a rural population in the Kilifi District of coastal Kenya, children (<13 years old) presenting during the peak of a RSV season in 2009 were screened for virus-associated ARI. Children were eligible if identified as having one or more of the following symptoms, unless the symptoms were deemed severe enough to require hospital referral: difficulty in breathing, nasal discharge, blocked nose, cough, or fast breathing for age. Informed consent was sought from the parent/guardian of each child. Ethical approval for the study was obtained from the Kenya National Ethical Review Committee. Three trained field assistants participated in this study. For each child, a field assistant collected a NFS specimen from one nostril, followed immediately with a NW sample from the other nostril. Thereafter, the caretaker (and children aged 3 years and above) responded to simple questions about their preferred specimen collection method. The two specimens were stored in a cool box, with ice packs, and transferred within 1 h to a refrigerator at ~4°C. Samples were transported in a cool box at the end of every day to the laboratory at KEMRI-Wellcome Trust Research Programme in Kilifi town.

NFS specimens were collected as described elsewhere (4) using a commercially available device that has a fine nylon flock on the tip of a flexible plastic rod (catalog number 503CS01; Copan, Italia). Briefly, the swab was gently passed up the nostril toward the pharynx for a distance equal to that between the patient's nares and earlobe, rotated 2 to 3 times, held in place for 5 s, withdrawn gently, and put in 2 ml of viral transport medium, which was locally prepared as described elsewhere (8, 15). For NW, normal saline (3 to 10 ml, according to age) was squirted into the patient's nasal cavity using a soft rubber bulb and immediately sucked out. Fluid escaping from the other nostril was collected in a suitable receptacle. The NW process was repeated in the other nostril if less than 1 ml of fluid was retrieved (7, 12). The two procedures were performed while the child was in a sitting position, head slightly tilted backward, with or without support from their caretaker.

NW samples were screened for RSV by a commercial IF kit (catalog number 3125; Millipore Light Diagnostics, Temecula, CA) as previously described (12), and if found positive, the paired NFS sample was also screened by IF. Slides for IF were prepared using a cytology centrifuge (Cytospin 3; Thermo Shandon Ltd., Cheshire, United Kingdom) (13). For all pairs of NW/NFS samples, nucleic acid was extracted using the QIAamp viral RNA minikit (catalog number 52906; Qiagen, United Kingdom) and tested by the M-PCR method, using the ABI-7500 platform (Applied Biosystems, Inc., CA), as described elsewhere (6) for 16 respiratory pathogens, namely, RSV subgroup A (RSV-A), RSV-B, adenovirus, rhinovirus, human metapneumovirus (hMPV), human coronavirus (NL63, OC43, 229E), parainfluenza (PIV; types 1, 2, 3, and 4), influenza (A, B, C), and *Mycoplasma pneumoniae.* Specimens were considered positive for a particular pathogen if the cycle threshold (C_T) value was ≤35.0; otherwise, they were consid-

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TABLE 1. Sensitivity of real-time multiplex PCR for detection of respiratory viruses in nasal wash versus posterior nasopharyngeal flocked swab collections

Respiratory virus (n = 299)[a]	No. of viruses detected by:				% sensitivity (95% CI)[b]		P value[d]
	Both NW and NFS	NW only	NFS only	Either NW or NFS[c]	NW	NFS	
Rhinovirus	50	8	21	79	73.4 (62.2-82.7)	89.9 (81.0-95.5)	0.024
RSV	61	4	9	74[e]	87.8 (78.2-94.3)	94.6 (86.7-98.5)	0.146
RSV-A	26	4	4	34	88.2 (72.5-96.7)	88.2 (72.5-96.7)	1
RSV-B	35	0	5	40	87.5 (73.2-95.8)	100 (91.1-100)	0.063
PIV	25	7	5	37[f]	86.5 (71.2-95.5)	81.1 (64.8-92.0)	1
Adenovirus	7	3	12	22	45.5 (24.4-67.8)	86.4 (65.1-97.1)	0.035
Coronavirus	16	3	3	22	86.4 (65.1-97.1)	86.4 (65.1-97.1)	1
hMPV	12	1	2	15	86.7 (59.5-98.3)	93.3 (68.1-99.8)	1
Total[g]	172	26	52	250	79.2 (73.6-84.1)	89.6 (85.1-93.1)	0.0043

[a] PIV, parainfluenza virus types 1, 2, 3, and 4.
[b] One-sided 97.5% CI reported if sensitivity was 100%.
[c] Virus positive by either NW or NFS was considered the true positive for the sensitivity analysis.
[d] Exact McNemar's significance probability values comparing sensitivities for NW and NFS.
[e] Includes one coinfection of RSV-A and -B.
[f] Includes one coinfection of PIV type 3 (PIV3) and PIV4.
[g] Shows all viruses detected, which includes one influenza A infection detected by both NFS and NW.

ered negative (2). The laboratory technicians were blinded to specimen pairing.

The sensitivity of NFS collection in the detection of RSV by IF was determined using NW IF results as the reference. For M-PCR data, a sample was considered a true positive if either of the specimens was positive, and comparisons were made using McNemar's chi-square test. The binomial exact method was used to determine 95% confidence limits for prevalence and sensitivities (one-sided 97.5% reported if sensitivity was 100%). The mean (95% confidence interval [CI]) of the C_T values by specimen collection method was calculated, and comparisons were made using paired t test for each virus. Each comparison was limited to sample pairs for which either sample had a C_T value of ≤35.0: "undetermined" C_T values (negatives) were coded as 40 for this analysis. Statistical analyses were done using STATA 11.1 (Stata-Corp LP, College Station, TX).

A total of 299 children had paired NW and NFS samples collected between 28 January 2009 and 17 April 2009. The median age (interquartile range) was 1.8 (0.9 to 4.2) years, with infants (<1 year of age) accounting for 89 (29.8%) of the samples. There were 145 male participants (48.5%).

There were 43 (14.4%) NW specimens positive for RSV by IF, and all 43 were also positive by IF for the paired NFS collections. The number of RSV positives detected increased to 64 (21.4%) and 70 (23.4%) by M-PCR for NW and NFS, respectively. Overall, 199 (66.6%) children had at least one virus detected from either NW or NFS by M-PCR, with 12 being detected from NW only and 30 from NFS only. The proportion of individuals positive for at least one virus was higher in NFS than in NW (187/299, 62.5% [95% CI, 56.8 to 68.0] and 169/299, 56.5% [95% CI, 50.7 to 62.2], respectively; McNemar's chi-square test, $P = 0.008$). Rhinovirus was the most frequently detected virus (79, 26.4%), followed by RSV (73, 24.4%), PIV (36, 12.0%), adenovirus (22, 7.4%), human coronaviruses (22, 7.4%), and hMPV (15, 5.0%). Influenza (A) virus was detected in one patient, in both the NFS and NW specimens. There was no detection of influenza B and C, human coronavirus 229E and OC43, or *Mycoplasma pneumoniae*. The sensitivities of the M-PCR for detection of respiratory viruses in NW and NFS are shown in Table 1. A total of 172 viruses were detected from both the NW and NFS collections, while 26 viruses were detected from NW only and 52 from NFS only by M-PCR. The sensitivity of NW and NFS in the detection of respiratory viruses by M-PCR was 198/259 (79.2%; 95% CI, 73.6 to 84.1) and 224/250 (89.6%; 95% CI, 85.1 to 93.1), respectively (McNemar's chi-square test, $P = 0.0043$).

A total of 74 RSV infections were detected by either IF or M-PCR assay. One participant had both samples positive by IF but all negative by M-PCR assay. The sensitivity for detection of RSV was higher for NFS (94.6% [95% CI, 86.7 to 98.5]) and NW (87.8% [95% CI, 78.2 to 94.3]) by M-PCR assay relative to NW IF (58.1% [95% CI, 46.1 to 69.5%], $P < 0.001$). Though there was no statistically significant difference in M-PCR RSV sensitivity using NFS rather than NW, the mean RSV C_T values were significantly lower (i.e., an indication of higher viral load) in NFS collections relative to those of NW, and this was the case for rhinovirus and adenovirus (P values < 0.05.) However, these differences were in the 1 to 2 C_T range (data not shown).

Of the 275 caretakers and 153 participants who responded to questionnaires on the acceptability of the specimen collection methods, 60.0% and 71.9% preferred NFS, 35.3% and 22.2% preferred NW, and there was no preference for either method by 4.7% and 5.9%, respectively. The 3 trained field assistants participating in this study preferred using NFS over NW in 80.2% of the 268 collections for which data were recorded. In all instances described above, there was evidence of a statistically significant preference for NFS over NW ($H_0 = 50\%$, where H_0 is the null hypothesis; Pearson's chi-square test, $P < 0.02$).

We found no evidence of inferiority of the NFS compared to the NW for the detection of RSV by IF. Relative to IF, the use of M-PCR significantly increased the proportion of RSV-positive cases (from 14% to ~22% of 299 participants) but with no marked difference between collection device. The sensitivity of

NFS was significantly higher than that of NW (89.6% versus 79.2%; $P = 0.0043$) for the detection of at least 1 of the 16 respiratory pathogens tested in the children with ARI who were managed as outpatients. Individually, the detection of rhinovirus and adenovirus using NFS had a statistically higher sensitivity relative to using NW, and this was reflected in lower (improved) C_T values in NFS relative to NW. This could be attributed to greater collection variability associated with NW and a dilution effect of saline in NW. NFS have been previously shown to yield adequate numbers of respiratory epithelial cells for the detection of viruses (4). This evidence suggests that NFS is a suitable alternative sampling device for detection of viruses as reported elsewhere (4, 9). NFS sensitivity estimates of above 90% for a range of viruses have been reported in a study comparing NFS and NPA using M-PCR assays (4). However, conflicting findings were reported when use of pernasal (shallow) flocked swabs and NPA were compared in immunocompromised patients (14). Posterior nasopharyngeal sampling seems to be a prerequisite to achieving comparable sensitivities to NPA or NW.

We conclude that NFS collection offers a suitable alternative to NW collection based on performance and acceptability for the detection of RSV by IF and for the detection of common respiratory viruses in general by M-PCR.

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REFERENCES

1. **Abu-Diab, A., et al.** 2008. Comparison between pernasal flocked swabs and nasopharyngeal aspirates for detection of common respiratory viruses in samples from children. J. Clin. Microbiol. **46:**2414–2417.
2. **Ahmed, A., M. F. Engelberts, K. R. Boer, N. Ahmed, and R. A. Hartskeerl.** 2009. Development and validation of a real-time PCR for detection of pathogenic leptospira species in clinical materials. PLoS One **4:**e7093.
3. **Berkley, J. A., et al.** 2010. Viral etiology of severe pneumonia among Kenyan infants and children. JAMA **303:**2051–2057.
4. **Chan, K. H., J. S. Peiris, W. Lim, J. M. Nicholls, and S. S. Chiu.** 2008. Comparison of nasopharyngeal flocked swabs and aspirates for rapid diagnosis of respiratory viruses in children. J. Clin. Virol. **42:**65–69.
5. **Forgie, I. M., et al.** 1991. Etiology of acute lower respiratory tract infections in Gambian children. I. Acute lower respiratory tract infections in infants presenting at the hospital. Pediatr. Infect. Dis. J. **10:**33–41.
6. **Gunson, R. N., T. C. Collins, and W. F. Carman.** 2005. Real-time RT-PCR detection of 12 respiratory viral infections in four triplex reactions. J. Clin. Virol. **33:**341–344.
7. **Hall, C. B., and R. G. Douglas, Jr.** 1975. Clinically useful method for the isolation of respiratory syncytial virus. J. Infect. Dis. **131:**1–5.
8. **Hammitt, L. L., et al.** 2011. Added value of an oropharyngeal swab in detection of viruses in children hospitalized with lower respiratory tract infection. J. Clin. Microbiol. **49:**2318–2320.
9. **Macfarlane, P., J. Denham, J. Assous, and C. Hughes.** 2005. RSV testing in bronchiolitis: which nasal sampling method is best? Arch. Dis. Child. **90:** 634–635.
10. **Meerhoff, T. J., et al.** 2010. Detection of multiple respiratory pathogens during primary respiratory infection: nasal swab versus nasopharyngeal aspirate using real-time PCR. Eur. J. Clin. Microbiol. Infect. Dis. **29:**365–371.
11. **Nair, H., et al.** 2010. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet **375:**1545–1555.
12. **Ngama, M. J., B. Ouma, M. E. English, and D. J. Nokes.** 2004. Comparison of three methods of collecting nasal specimens for respiratory virus analysis. East Afr. Med. J. **81:**313–317.
13. **Nokes, D. J., et al.** 2008. Respiratory syncytial virus infection and disease in infants and young children observed from birth in Kilifi District, Kenya. Clin. Infect. Dis. **46:**50–57.
14. **Ohrmalm, L., et al.** 2010. Flocked nasal swab versus nasopharyngeal aspirate for detection of respiratory tract viruses in immunocompromised adults: a matched comparative study. BMC Infect. Dis. **10:**340.
15. **WHO.** 2006. Collecting, preserving and shipping specimens for the diagnosis of avian influenza A(H5N1) virus infection: guide for field operations. Annex 8: viral transport media (VTM), p. 42. http://www.who.int/csr/resources/publications/surveillance/WHO_CDS_EPR_ARO_2006_1.pdf.